Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanospacings

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Keywords: stem cell, nanopattern, cell differentiation, cell adhesion, cell proliferation, RGD, PEG hydrogel

Abbreviations: RGD, arginine-glycine-aspartate; PEG, poly(ethylene glycol); MSC, mesenchymal stem cells; ECM, extracellular matrix; AFM, atomic force microscope; ALP, alkaline phosphatase; DAPI, 4',6-diamidino-2-phenylindole; PS-b-P2VP, polystyrene-block-poly(2-vinylpyridine); F-actins, filamentous actins; CCD, charge coupled device

The present report is an extension of our preceding publication in Biomaterials (2013) entitled “Effect of RGD nanospacing on differentiation of stem cells.” Cell-adhesive peptide arginine-glycine-aspartate (RGD) was nanopatterned on a non-fouling poly(ethylene glycol) (PEG) hydrogel, and mesenchymal stem cells (MSCs) derived from rat bone marrow were cultured on the patterned surfaces at nanospacings from 37 to 124 nm. Cell adhesion parameters such as spreading areas varied with RGD nanospacings significantly. The differences were well observed at both the first and eighth days, which confirmed the persistence of this nanospacing effect on our nanopatterns. The proliferation rate also varied with the nanospacings. Osteogenic and adipogenic inductions were undertaken, and a significant influence of RGD nanospacing on stem cell differentiation was found. The effect on differentiation cannot be simply interpreted by differences in cell adhesion and proliferation. We further calculated the fractions of single, coupled, and multiple cells on those nanopatterns, and ruled out the possibility that the extent of cell-cell contact determined the different differentiation fractions. Accordingly, we reinforced the idea that RGD nanospacing might directly influence stem cell differentiation.

Introduction

Stem cells play a vital role in organogenesis. In tissue engineering and regenerative medicine, stem cells are usually loaded into biomaterials instead of being used alone. Ideally, biomaterials should, to a certain extent, mimic the extracellular matrix (ECM) to promote cell adhesion, proliferation, and differentiation. Hence, interactions between cells and materials become a key fundamental topic. One of the core peptide sequences in ECM proteins is arginine-glycine-aspartate (RGD), which can be specifically bound to integrin, a receptor across the membrane. The RGD motif has been usefully employed in cell research and biomaterial modification.

Considering that the size of integrin is about 8 to 12 nm, patterns with RGD peptides grafted onto nanodots of about 10 nm diameter are important when investigating the effect of spatial distribution of RGD ligands on cell behavior. An ideal RGD nanopattern should be based upon a perfect non-fouling background. A hydrogel of poly(ethylene glycol) (PEG) affords as such a background, and a transfer lithography technique has been proposed to generate gold patterns on PEG hydrogels. After grafting RGD-thiol ligands onto gold domains embedded in the PEG hydrogel, RGD patterns on a non-fouling background were obtained.

Although cell adhesion and migration on RGD nanopatterns have been extensively investigated, the behaviors of stem cells on those patterns have not yet been reported until 2013. Our group examined osteogenic and adipogenic differentiations of mesenchymal stem cells (MSCs) derived from the bone marrow of rats and found an underlying nanospacing effect. A schematic presentation of this study is given in Figure 1. Novelty is claimed as follows: (1) besides cell differentiation, adhesion of stem cells will be analyzed, and besides average values, the distributions of spreading area, circularity, and aspect ratio of cells on nanopatterns of varied nanospacings will be reported; (2) proliferation rates of stem cells on RGD nanopatterns will be examined; (3) we will compile statistics about single, coupled, and multiple cells on nanopatterns, to check whether or not cell-cell contact on nanopatterns of varied nanospacings can account for the origins of the nanospacing effect on stem cell differentiation.

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Submitted: 04/28/13; Revised: 08/07/13; Accepted: 08/07/13
http://dx.doi.org/10.4161/org.26080

www.landesbioscience.com Organogenesis
Preparation of RGD Nanopatterns on PEG Hydrogels

First, gold nanopatterns were prepared by block copolymer micelle nanolithography, and characterized by atomic force microscopy (AFM) (Fig. 2). The average size of gold particles was measured using their heights as a proxy, resulting in approximately 10 nm.

Then, the gold nanoarrays were transferred to the surfaces of PEG hydrogels, which provide a non-adhesive background. Finally, the RGD motifs were grafted onto the gold particles by way of an S-Au bond to obtain nanopatterns of RGD motifs. Since the size of an integrin molecule is about 10 nm, this technique might achieve a one-to-one correspondence between a nanodot and an integrin receptor. The RGD patterns can thus regulate the spatial distribution of their receptor integrins.

Strong and Persistent Non-fouling of the PEG Hydrogel in Cell Culture Medium

The process of cell adhesion includes specific, and non-specific, adhesion. To guarantee that cells adhere to the RGD nanopatterned surfaces mainly through bioconjugation of RGD ligands to integrins across the cell membrane, namely, specific adhesion, one should first confirm the persistent resistance of the non-specific adhesion of the substrate background. However, ensuring non-fouling conditions in the culture medium with serum for several days presents technical difficulties.

Herein, we took advantage of the strong and persistent non-fouling property of PEG hydrogels, which has been confirmed both in vitro and in vivo. The persistent non-fouling property of the nanopatterned substrate could be well reflected by avoiding cell adhesion beyond what we termed the dip-line in our experiments. Here the dip-line is the border between regions with and without gold nanoarrays formed during the dip-coating process, in which glass coverslips were dipped into, and then pulled out of, a solution of block copolymer micelles loaded with HAuCl₄. The resulting substrate surface thus contained a nanopatterned region and a non-nanopatterned region separated by the dip-line. Fluorescence images of cells revealed that cells preferred to adhere to the nanopatterned region (Fig. 3). Even after eight days of cell culture, MSCs still stayed inside the patterned region and rarely migrated to the unpatterned region. It indicated that the PEG hydrogel afforded a strong and persistent anti-adhesive background, and therefore cells were immobilised on the RGD-nanopatterned region just by bioconjugation between RGD ligands on the substrate and integrins in the cell membrane.

Distributions of Adhesion Parameters of Stem Cells on RGD Nanopatterns

As the RGD nanospacing increased, the mean of the spreading area of the stem cells decreased, the mean cell circularity increased, and the mean cell aspect ratio did not change in any regular way according to our previous report. Herein, their distributions were further calculated. We measured over one hundred cells from each nanopattern of different nanospacings using an image processing program (ImageJ, freely available from: www.nih.gov). The resultant distributions of cell spreading parameters are shown in Figure 4. The peak values of cell area, cell circularity, and aspect ratio changed with the same trend as their average values. The effect of RGD nanospacing on stem cell adhesion was thus strengthened.

Proliferation of Stem Cells on RGD Nanopatterns

While all previous studies of non-stem cells on RGD nanopatterns were examined within 24 h, we checked the adhesion until the eighth day, by which time there were large numbers...
Cell Adhesion and Clustering Do Not Fully Explain the Nanospacing Effect on Cell Differentiation

It has been known from our examination of MSCs on microscale RGD patterns that adhesion parameters including cell density, spreading areas, and cell shape can influence cell differentiation. These factors have been excluded as the reasons to account for the nanospacing effect on differentiation of MSCs according to our previous analysis.

Besides adhesion parameters, cell-cell contact has also been recognized as regulating stem cell differentiation. Herein, cells on patterns of large RGD nanospacings did not spread well and may even shrink and aggregate into cell clusters. So the extent of cell-cell contact changed with RGD nanospacing. We counted the numbers of isolated single cells and clustered cells that came into contacted with others. The statistical results indicated that the fraction of single and coupled cell numbers increased with the RGD nanospacing, the fraction of clusters of cells. Some of cells even peeled off if overcrowded, which led to difficulty when examining cell differentiation. So, we added a cell-division inhibitor (aphidicolin, 0.5 μg/mL) to our cell culture. Nevertheless, cell proliferation could still be observed, as seen from the comparison between densities of cells cultured in growth medium for 8 d (GM 8 d) and 1 d (GM 1 d), and also from the corresponding data in the insert to Figure 5. The proliferation rate decreased slightly with RGD nanospacing except for a significant increase at very large nanospacings, where the low initial density of the adherent cells may play a role.

The presence of induction agents slowed down cell proliferation, as also seen in Figure 5. The trend is consistent with the well-known principle that a stem cell does not proliferate and undergo differentiation simultaneously. The proliferation rates were quite small in presence of both induction medium and 0.5 μg/mL aphidicolin, which made the forthcoming examination of cell differentiation less subject to interference and thus more convincing.

Differentiation of Stem Cells on RGD Nanopatterns of Different Nanospacings

After one day of adhesion in growth medium, osteogenic, and adipogenic inductions of stem cells lasted for 7 d. Cells were then stained to enable later counting to establish the extent of differentiation. Osteoblasts expressing alkaline phosphatase (ALP) were stained in blue, and adipocytes in red. Due to space limitations we do not show all images of the stained cells experiencing osteogenic or adipogenic induction on different RGD nanopatterns. Typical images of stained cells on nanopatterns with RGD nanospacings of 37 nm and 87 nm are shown in Figure 6. Relatively more stem cells were committed to osteoblasts and adipocytes on 37 nm-spaced nanopatterns than on 87 nm-spaced nanopatterns. The differentiation fractions at different nanospacings have been reported elsewhere. Larger RGD nanospacings induced greater extents of both osteogenic and adipogenic differentiation.
both osteogenic and adipogenic differentiations, the increase of nanospacing enhanced the two lineage commitments. Thus the change of cell-cell contact extent cannot explain the differentiation trend of stem cells. The present study implies that the RGD containing three or more cells decreased, and cell clusters were predominant under our experimental conditions (Fig. 7). So, the extent of cell-cell contact became weaker with the increase of RGD nanospacing. While cell-cell contact is beneficial for

Figure 4. Distributions of cell area, cell circularity, and cell aspect ratio on patterned surfaces of RGD peptides on the PEG hydrogels with indicated nanospacings. The means and standard deviations are marked inside the graphs.
Preparation and characterization of nanopatterns. Gold nanopatterns on glass surfaces were prepared following our previous protocol. Before being transferred to PEG hydrogels, the gold nanoarrays on glass were characterized with AFM (Multimode IV, Bruker) under tapping mode. For each group of patterns of a given nanospacing, six samples were randomly selected from the population of 100 samples. For each sample, three regions were randomly selected for scanning, and the nanodot spacings were measured and averaged. Before cell culture, c(-RGDfK)-thiol ligands (f: d-phenylalanine, K: l-lysine; Peptides International) were grafted onto gold nanodots.

Culture and induction of stem cells. Primary MSCs were isolated from marrows of tibia and femur of 7-d Sprague-Dawley (SD) rats. The MSCs of the second passage were seeded on RGD nanopatterned surfaces at a density of 6600 cells per cm². We cultured the cells in the growth medium (GM) for both 1 d and 8 d to test cell densities and spreading parameters. The GM was composed of low-glucose Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). In the process of 8-d culture, the culture medium after cells adhered in the GM for 1 d. The following induction lasted for 7 d. The osteogenic induction medium (OM) was composed of high-glucose DMEM, 10% FBS, 50 μM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, and 100 nM dexamethasone (Sigma). The OM was changed on the third and fifth days of induction. The adipogenic induction medium (AM) was composed of high-glucose DMEM, 10% FBS, 1 mM dexamethasone, 200 mM indomethacin, 10 mg/mL insulin, and 0.5 mM methylisobutylxanthine (Sigma). On the fourth and fifth days of the adipogenic induction, cells were cultured in a maintenance medium composed of high-glucose DMEM, 10% FBS, and 10 mg/mL insulin. In all cases, aphidicolin was added from the fourth to fifth day of cell culture or cell induction to diminish interference with cell proliferation.

Staining of cells and statistics of differentiation extents. After 1 d and 8 d of culture, cytoskeletons and nuclei of cells were fluorescently stained, with F-actins labeled by phalloidin-TRITC (Sigma) and nuclei by 4’,6-diamidino-2-phenylindole (DAPI, Sigma). The positively stained cells were counted. Then, the osteogenic and adipogenic differentiation extents of MSCs on nanopatterned surfaces were calculated.

Statistics of cell densities and spreading parameters. Cell densities were obtained by counting the numbers of cell nuclei in fluorescence images of cells stained by DAPI. Cell areas, aspect ratios (ARs), and circularities were measured by outlining cells in fluorescence images with ImageJ (freely available from: www.nih.gov).

In conclusion, a series of nanopatterns of RGD ligands were fabricated on a non-fouling PEG hydrogel: stem cells on the patterns of varied RGD nanospacings exhibited different adhesion, proliferation, and differentiation behaviors. The increased nanospacing led to a smaller cell density and less cell spreading. Cell proliferation decelerated in the presence of the induction agent and division inhibitor. A larger RGD nanospacing was beneficial for both osteogenesis and adipogenesis of MSCs. The differences in cell differentiation can be explained by neither cell spreading, shaping, adherent density, nor cell clustering. RGD nanospacing may directly regulate cell differentiation.

The universality of this principle is ready to be checked by examination of other stem cell types and even some non-stem cells with differentiation abilities such as pre-osteoblasts. The mechanism of the nano-phenomenon remains a challenging topic. The nanospacing effects on cell adhesion, proliferation, and differentiation are potentially stimulating for the precise design of new-generation biomaterials.
AR is defined as the ratio between the two principal axes, which were obtained from the two eigenvalues of the corresponding cell profile calculated by the ImageJ software. Circularity is quantified by area multiplied by $4\pi$ divided by the square of the perimeter.

Calculation of proliferation rates. The proliferation rate or growth rate $G$ was reflected simply by cell density at day 8 ($D_8$) over that at day 1 ($D_1$).40,41 The error bound on $G$ was obtained by the following transfer formula

$$
\frac{\delta G^2}{G^2} = \frac{\delta D_8^2}{D_8^2} + \frac{\delta D_1^2}{D_1^2}
$$

where $\sigma$ denotes the corresponding standard deviation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful for financial support from The Chinese Ministry of Science and Technology (973 Program No. 2009CB930000 and No. 2011CB606203), The NSF of China (Grants No. 21034002, No. 91127028, and No. 51273046), and The Science and Technology Developing Foundation of Shanghai (Grant No. 13XD1401000).